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ORIGINAL PAPER

Morphometric discrimination of wild from farmed Dybowski's frog (*Rana dybowskii*) based on hindlimb length

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Abstract: Commercial farming of anuran species that are declining in the wild raises a need to discriminate wild from farmed frogs. We hypothesized wild frogs might have extended hindlimbs due to greater frequency or intensity of jumping relative to farmed frogs, highlighting a morphometric approach to discrimination of wild from farmed frogs using hindlimb length. In the present study, Dybowski's frog (*Rana dybowskii*) was used to test this hypothesis. We measured body mass (M_b) and hindlimb length (L_h) of 2-year old farmed frogs and wild frogs aged 2 to 5 years. Dybowski's frog demonstrated significant dimorphism in M_b and L_h. M_b was significantly greater among farmed 2-year old frogs in both sexes (p=0.000), while only among females was L_h significantly greater for wild frogs (p=0.000). L_h/M_b was used as an index for origin discrimination to eliminate the influence of M_b due to variation of husbandry conditions among farms. Mean L_h/M_b for farmed frogs was significantly lower than for wild frogs (p=0.000) in the 2-year old age class. Discrimination correctly classified 84.4% of farmed and 96.3% of wild male frogs. Among females, 92.9% of farmed frogs and 90.1% wild frogs were correctly classified. The overall correctness of classification was 92.1% and 90.8% for males and females, respectively. However, L_h/M_b revealed variation with age, resulting in reduced discriminative power for frogs ≥3 years old. We introduced a coefficient C_e to adjust the L_h/M_b of frogs ≥3 years to the level equivalent to 2-year frogs. The adjustment achieved 89.5% for overall correctness of origin for wild males and 92.4% for wild females ≥3 years old. These results show that L_h/M_b is an effective index to discriminate wild from farmed Dybowski's frog. Since the physical demands of jumping are common among anurans, this index is also potentially applicable to other anuran species.

Key words: Dybowski's frog; morphometric discrimination; wild, farmed;, *Rana dybowskii*

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Introduction

Humans have always eaten wild animals. However, wildlife has been depleted worldwide and is no longer adequately abundant to serve as a human food resource in most of the world. To compensate for this shortage, wildlife farming has been adopted for some high-value species. Many wildlife species have been domesticated and farmed successfully, including fur-bearing mammals, snakes, frogs, crocodiles, ostriches, deer, giant rat, snail, geese, and ducks. Many farmed wildlife species are endangered in the wild and protected by law or regulation. However, their wild populations are still occasionally harvested or poached and these wild animals enter food markets alongside their farmed conspecifics. This raised the question of how to distinguish the wild animals from those reared on farms.

One approach to discrimination of wild from farmed animals is based on differences between the wild and farm environments and the impacts of these differences on animal physiology. It is widely accepted that morphology and physiological processes evolve in response to environmental stimuli to enhance individual survival and reproductive success. In contrast to nature, the captive environment is confined, requires lower activity levels, is limited in terms of diet, requires less energy expenditure to procure food, and has a lower predation risk (McPhee 2004). Captive animals respond to this less demanding environment through physiological and morphological evolution that results over time in phenotypic divergence from their wild conspecifics (McPhee

2003a; McPhee 2003b). These evolutionary changes can point researcher and law enforcement personnel toward better approaches to segregating wild from farmed animals.

Some researchers developed analytic methods based on stable isotope deposition in hard tissues such as fur, claws, and teeth (Hammershøj et al 2004). Carbon ratios ($^{13}C/^{12}C$) have been used to distinguish farmed from wild animals based on differences in their food sources (Chisholm et al 1982; Kelly 2000; Smith et al 1996; Knoff et al 2002). A similar approach is based on the differences in fatty acid profiles between wild and captive animals due to differences in sources of dietary fat (Rouvinen et al 1989). However, both stable isotope content and fatty acid profiles may change in response to changes in diet (Ostwald 1962; Knoff et al 2002), thus are only applicable for special situations. Bone structure and density are associated with diet and activity. Animals in the wild tend to be more active than those in the farm; therefore, femur bone density is a stable index useful for discriminating wild and farmed animals (Yang et al. 2011). However, these approaches require bones to be isolated from the test animals and are often impracticable for use on live animals.

The provision of an adequate, readily obtainable diet together with protection from potential predators and provision of confined living spaces would cause captive animals to spend significantly less energy than wild animals on feeding, foraging, traveling and vigilance. As a result, the body fat mass of captive animals should be higher, while activity and motion intensity should be lower than among wild animals.

For frogs, jump distance and frequency is determined by the structure and function of the hindlimbs (Peplowski et al. 1973). Jumping is a physical factor stimulating the development of hindlimbs of frogs (Olson et al. 1998.) because it positively impacts development of bone and muscle (Watanabe et al. 2007; Gary et al. 2001). As a result, hindlimbs of wild frogs can be considered optimally developed to provide leaping power needed for survival in the wild (Essner et al 2010; Leisler 1977). This suggests that metrics of the hindlimbs of frogs could provide a novel approach to discriminate wild from farmed frogs because frogs living in the wild could have longer hindlimbs than their counterparts who have lived many generations in farms.

Dybowski's frog (*Rana dybowskii*) occurs in northeast China, Mongolia, Russian Far East, Korean Peninsula, and Japan (Xie et al. 1999). The extensive use by humans of dried frog oviduct as a natural invigorant causes unsustainable taking of Dybowski's frog from the wild and this has resulted in declines in wild populations (Liu et al. 2007). As wild populations declined, market prices rose, and this stimulated captive breeding trials (Li et al. 1987; Meng et al. 1990). Dybowski's frog farms were established after the mid 1980s throughout the species range in northeast China (Liu et al. 2007). Frogs are commonly kept in pens as small as 200-1000 m^2 and surrounded by the fences (Bai et al. 2006.). They are fed a limited range of readily available invertebrate species, such as yellow meal worm, screwworm, and earthworm. We hypothesized that these living conditions would result in changes in the morphology of frogs, specifically a reduction in the ratio of hindlimb length to body mass, that would enable discrimination of farmed from wild frogs. We used Dybowski's frog to test this hypothesis.

Materials and methods

A total of 335 Dybowski's frogs were captured in the Jiaohe (126°53′ E, 44°02′N, altitude 244 m), Fusong (127°33′ E, 42°21′ N, altitude 780m), and Tonghua (126°12′ E, 42°03′ N, altitude 632 m) regions of China's Changbai Mountains in October 2006, including 182 females and 173 males. Age of each frog was determined by the tibiofibula slice procedure as described in Yang et al (2011). 60 frogs (32 males and 28 females) at the age of two years were collected from a frog farm located in the same region (126°29′ E, 44°10′ N, altitude 174 m) where the wild frogs were captured. The frogs were raised in 5 m×10 m pens after metamorphosis with density varying from 100–200 frogs per square meter. The frogs were harvested before winter hibernation at the age of two years, usually during September and October.

Body mass (M_b) of these frogs was measured using an electronic balance with 0.01g sensitivity (Shanghai Yueping Instrument Co. Ltd., China). Hindlimb length (L_h) was measured from the hind end of the body to the tip of the fourth toe. L_h measurements were preformed using a screw thread micrometer calliper with accuracy of 0.1mm.

A relative index, L_h/M_b, was computed for each sex and age group of wild and farmed frogs. Variation of L_h/M_b with age and geographic location was examined using ANOVA multiple comparisons (Tamhane method). Mean L_h/M_b values were compared between farmed and each of three wild populations of 2-year old frogs for each sex using ANOVA multiple comparisons (Tamhane method). We further pooled the data of three wild populations and compared L_h/M_b as a whole population to farmed population for each sex using nonparametric Mann-Whitney U tests (two-tailed) at significance level α=0.05.

In order to improve the power of discrimination for frogs older than two years, the relationship between age and M_b was estimated for the pooled wild populations for each sex using the curve estimation method of regression. The regression function was used to estimate the age of a given wild frog by using its value of M_b. A coefficient C_e was calculated as the mean L_h/M_b of 2 year frogs divided by mean L_h/M_b of 3 year, 4 year and ≥5 year frogs. Then, an adjusted L_h/M_b of the frog ≥ 3-year old was deduced through the observed L_h/M_b value multiplied by C_e. The deduced L_h/M_b was then used for origin discrimination. Finally, we tested the effectiveness of the deduced L_h/M_b in discrimination of wild frogs ≥ 3-year old from 2-year old farmed frogs using discrimination analysis. All statistical analyses were performed using SPSS16.0 software (SPSS Inc., Chicago, IL, USA).

Results and discussion

Origin discrimination of 2-year old frogs

We collected 172 two-year old wild frogs, 52 in Tonghua including 23 males and 29 females, 59 in Jiaohe including 29 males

and 30 females, and 61 in Fusong including 29 males and 32 females. The number of 3-year old wild frogs was 60 including 30 males and 30 females. The number of 4-year old frogs was 61 including 30 males and 31 females. Frogs ≥ 5-year old included 32 males and 30 females, 62 individuals in total.

M_b of male farm frogs was 16.33 g on average with a standard deviation (SD) 3.27. Mean M_b of male wild frogs (pooled) was 11.65g with a SD 2.52, significantly lower than farmed individuals (p=0.000). M_b of female farm frogs was 20.55±3.98 g. M_b of female wild frogs (pooled) was 13.04±3.55 g, also significantly lower than farm frogs (p=0.000). L_h was 51.21±3.49 mm in male farm frogs and 51.04±3.55 mm in male wild frogs, and this difference was not significant (p=0. 791). Among females, L_h was 55.40±4.61 mm for farm frogs and 51.99±2.95 mm for wild frogs, a highly significant difference (p=0.000).

Means and standard deviations of the ratio L_h/M_b for wild and farmed frogs are shown in Table 1. L_h/M_b exhibited geographical variations in both male and female wild frogs. Among males, L_h/M_b did not differ between the Jiaohe and Fusong populations (P=0.989). However, L_h/M_b for the Tonghua population was significantly greater than for the Jiaohe and Fusong populations (p=0.000). Such geographical variations were also seen among females. The Fusong and Tonghua female frogs had similar L_h/M_b ratios (p=0.609). However, Jiaohe females had significantly lower L_h/M_b ratios than did females at Fusong and Tonghua (p=0.000).

Table 1 Average and standard deviation of L_h/M_b in wild and farmed frogs aged 2 years

Origin	Male	Female
Wild populations		
Jiaohe	4.139±0.518	3.299±0.267
Fusong	4.231±0.575	4.669±0.704
Tonghua	5.524±1.057	4.964±0.963
Pooled	4.565±0.942	4.312±1.003
Farmed population	3.099±0.406	2.746±0.377

Comparison of farmed with wild frogs showed that for both sexes, mean L_h/M_b for farmed frogs was significantly lower in all three wild populations (p=0.000). A similar result was obtained when data of three wild populations were pooled (p=0.000). Fig. 1 shows the frequency distribution of indexes between farmed and wild (pooled) frogs.

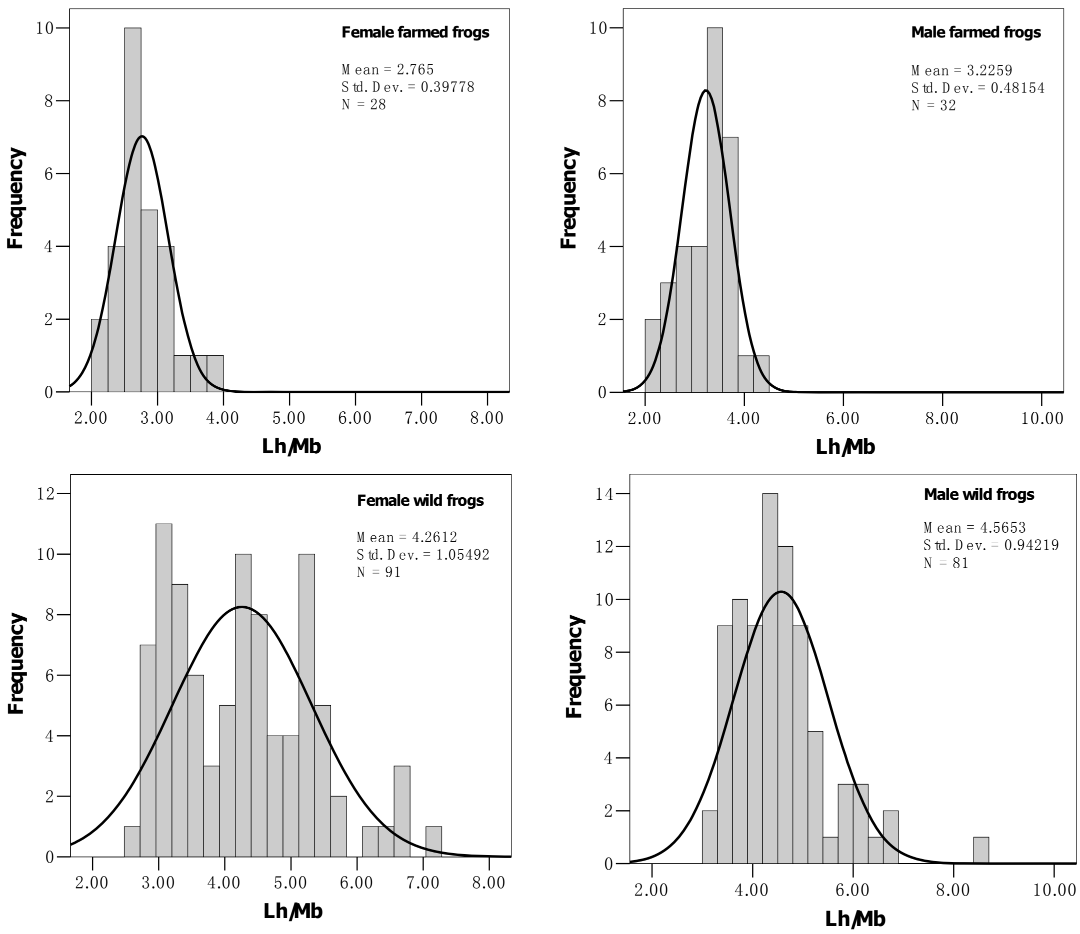

Fig. 1 Frequency distribution of L_h/M_b in 2-year farmed and wild Dybowski's frogs

Discrimination of 2-year old males using L_h/M_b correctly classified 84.4% of farmed frogs and 96.3% of wild frogs. Among females, 92.9% of farmed frogs and 90.1% wild frogs were correctly classified. The overall correctness of classification was

92.1% and 90.8% for males and females, respectively. The Fisher's linear discriminant function for male farmed frogs was

$$Y_1 = 4.519\, L_h / M_b - 8.264$$

and for male wild frogs was

$$Y_2 = 6.656\, L_h / M_b - 15.526$$

The function for female farmed frogs was

$$Y_1 = 3.404\, L_h / M_b - 6.121$$

and for female wild frogs was

$$Y_2 = 5.344\, L_h / M_b - 11.789$$

A frog could be classified as farmed if $Y_1 > Y_2$, otherwise it would be classified as wild.

Origin discrimination of ≥3-year old frogs

Variation of L_h/M_b with age of wild frogs is shown in Figure 2. In both male and female groups, L_h/M_b tended to decline with the age increment. Discrimination power between 2-year old farmed frogs and wild frogs older than 2 years resulted in 0 correctness for both male and female farmed frogs. M_b of frogs at the ages of 3 years, 4 years, and ≥5 years were similar (Figure 3). Therefore, frogs older than 2 years could be pooled to simplify discrimination. Pooling yielded two age classes for wild frogs, namely, 2 years and ≥3 years. We introduced an adjustment procedure: firstly, to assign frogs to an age class using M_b data; secondly to create a coefficient by which L_h/M_b values for frogs ≥3 years could be adjusted to levels equivalent to those of the 2-year age group; and thirdly to discriminate the origin of frogs using Fisher's linear discriminant functions.

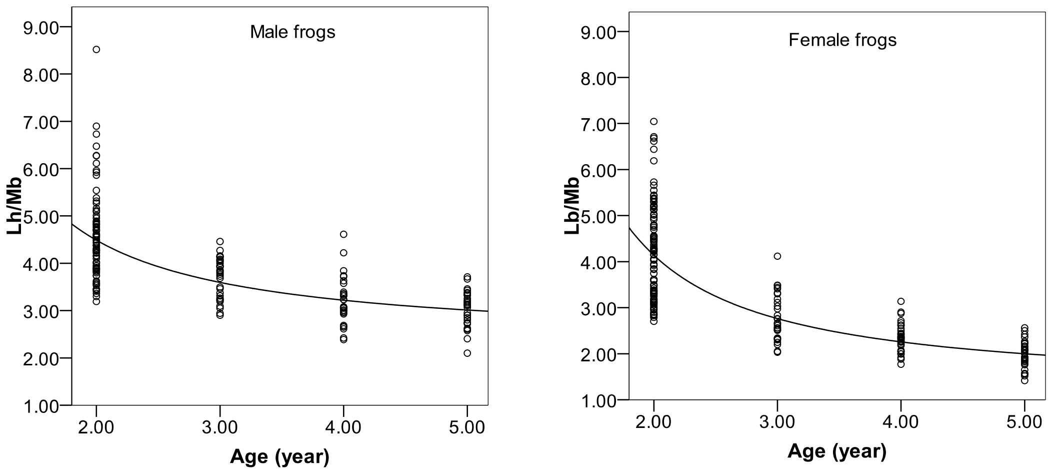

Fig. 2 Plots of L_h/M_b against age classes in wild Dybowski's frogs

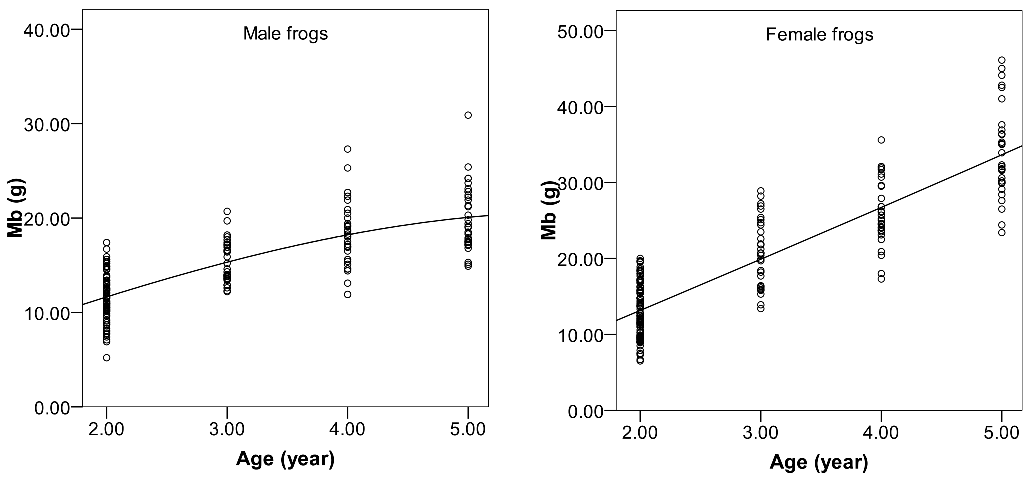

Fig. 3 Plots of M_b against age classes in wild Dybowski's frogs

We tested the effectiveness of the above procedure using our data for wild frogs. Assignment to age class can be performed through the discriminant functions for male wild frogs:

$$Y_1 = 1.146 M_b - 7.366$$

for group of 2 years, and

$$Y_2 = 1.762M_b - 16.476$$

for group of ≥3 years. The functions for females are:

$$Y_1 = 0.398M_b - 3.288$$

for group of 2 years, and

$$Y_2 = 0.821M_b - 11.720$$

for group of ≥3 years. An individual could be assigned to 2-year old group if Y1 > Y2, and otherwise to ≥3 years group.

The equivalent adjusting coefficient C_e of L_h/M_b was calculated as the ratio of the mean L_h/M_b of the 2-year group to the mean L_h/M_b of the ≥3-year group. The coefficient, C_e, we calculated was 1.382 for male frogs and 1.797 for female frogs.

Discriminant analysis showed 82.7% of 2-year old male frogs and 80.4% of ≥3 years old male frogs could be correctly assigned to age classes, with an overall correct percentage of 81.5%. The correct percentage for female assignment was 94.5% for 2-year old frogs, and 85.7% for ≥3 years old frogs, overall 90.1%. By adjusting with C_e, the mean L_h/M_b of male wild frogs ≥3-years old increased to 4.566±0.701 from 3.304±0.508, while the mean L_h/M_b of female frogs increased to 4.262±0.908 from 2.371±0.505.

Discrimination using the adjusted L_h/M_b values correctly classified 75.0% of 2-year old male farmed frogs and 94.6% of male wild ≥3-year frogs. Among females, 89.3% of 2-year old farmed frogs and 93.4% of wild ≥3-year frogs were correctly classified. The overall correctness of classification was 89.5% and 92.4% for males and females, respectively.

Discussion

Morphometric measurement does not injure the animals, thus is a convenient method to discriminate wild from farmed frogs. However, discriminating wild from farmed frogs as presented here is based on the measurement of morphological traits that are susceptible to modification by environmental conditions. Such traits must meet several criteria: (1) they must be in response to specific factors that are substantially different between captive and wild environments; (2) they must adequately plastic to permit partitioning of wild from captive specimens; and, (3) they must be characterized by low within-group variation so as to achieve high discriminative power in statistics.

Our hypothesis was that wild frogs would have extended hindlimbs. However, this hypothesis was poorly supported by our data for hindlimb length (L_h). Dybowski's frog demonstrated significant dimorphism that L_h was significantly greater for wild than farmed female frogs (p=0.000), while the difference was not significant (p=0.791) among males.

For males, M_b of farmed frogs was significantly greater than for wild frogs (p=0.000). For females, M_b was also significantly greater for farmed than wild frogs (p=0.000).

Considering the fact that the load on limbs during jumping and landing is associated with body mass, we used M_b as an objective reference to obtain a relative index L_h/M_b. Our results demonstrated L_h/M_b exhibited geographical variations in wild frogs. This demonstrates that frog growth is influenced by habitat conditions, and therefore absolute indexes might not be reliable indicators of frog origins. Despite the geographical variations found among the three sampled wild populations, L_h/M_b in 2-year old farmed frogs was significantly lower than that in each of the three wild populations at the same age for both sexes (p=0.000). A similar result was obtained when data for the three wild populations were pooled (p=0.000). These results suggest the index L_h/M_b is a useful indicator of origin for Dybowski's frog. Discrimination analysis showed an overall correctness of origin assignment of 2-year old frogs using L_h/M_b of 92.1% and 90.8% for males and females, respectively. We provided discriminant functions to perform the identification.

However, L_h/M_b revealed a notable variation with age (Figure 2), resulting in dramatically reduced discriminative power for frogs ≥3 years old. We introduced a coefficient C_e to adjust the L_h/M_b values of group of ≥3 years to the level equivalent to group of 2 years. This adjustment achieved 89.5% for overall correctness of origin identification for wild males and 92.4% for wild females ≥3 years old.

In conclusion, L_h/M_b is an effective index to discriminate wild from farmed Dybowski's frog. It should be noted that most anurans move by jumping and follow the same principle of hindlimb development. Therefore, L_h/M_b might also be applicable to similar investigations of other anurans.

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